Norgren, L., Weiss, N., Nikol, S., Hinchliffe, R., C Lantis, J., Patel, M. R., Reinecke, H., Ofir, R., Rosen, Y., Peres, D., & Aberman, Z. (2019). PLX-PAD Cell Treatment of Critical Limb Ischaemia: Rationale and Design of the PACE Trial. *European Journal of Vascular and Endovascular Surgery*, *57*(4), 538-545. https://doi.org/10.1016/j.ejvs.2018.11.008

PLX-PAD Cell Treatment of Critical Limb Ischemia – Rationale and Design of the PACE trial

Lars Norgren [a], Norbert Weiss [b], Sigrid Nikol [c], Robert J Hinchliffe [d], John C Lantis [e], Manesh R Patel [f], Holger Reinecke [g], Racheli Ofir [h], Yael Rosen [h], Dan Peres [h], Zami Aberman [h]

a. Department of Surgery, Faculty of Medicine and Health, Örebro University, Sweden
b. University Center for Vascular Medicine and Department of Medicine - Section Angiology, University Hospital Carl Gustav Carus at the Technische Universität Dresden, Germany
c. Asklepios, Klinik St Georg, Hamburg, Germany
d. Bristol Centre for Surgical Research, Bristol NIHR Biomedical Research Centre, University of Bristol, Bristol UK
e. Icahn School of Medicine, New York, USA
f. Duke University, Durham, NC, USA
g. Department of Cardiology I – Coronary and Peripheral Vascular Disease, Heart Failure, University Hospital of Muenster, Muenster, Germany
h. Pluristem Ltd., Haifa, Israel

Correspondence: Professor Lars Norgren, Department of Surgery, University Hospital, S-70185 Örebro, Sweden.
e-mail lars.norgren@regionorebrolan.se

CLINICALTRIALS.GOV: NCT03006770

Short title: PLX-PAD cell CLI trial design

Word count: 3976

Intended as original article

***What this paper adds*:**

Placebo controlled trials of cell therapy to reduce major amputations in patients with critical limb ischemia and no-option for revascularization have so far not been successful. PLX-PAD cell treatment (placenta derived adherent stromal cells) has in small studies shown promising results, and the phase III PACE trial is designed to evaluate the efficacy and safety of two sessions of intramuscular injections, 8 weeks apart in follow up of 12-36 months. Thus, the study will provide long term outcome and will collect parameters to assess potential ecomomic benefit for this kind of treatment.

Abstract

Background: Critical limb ischemia (CLI) is a life threatening condition with a considerable risk for death and major amputation. Besides revascularization, no treatment has been proven to reduce the risks. Therapeutic angiogenesis by gene or cell therapy has not demonstrated definitive evidence in randomized controlled trials. PLX-PAD is an 'off-the-shelf' allogeneic placental derived, mesenchymal-like cell therapy that in preclinical studies has shown pro-angiogenic, anti-inflammatory and regenerative properties. Favorable 1-year amputation free survival (AFS), and trends in reduction of pain scores and in increase of tissue perfusion have been shown in two small, open-label, phase I trials.

Study design: The PACE study is a phase III randomized, double-blind, multicenter, multinational placebo-controlled, parallel-group study to evaluate the efficacy, tolerability and safety of intramuscular injections of PLX-PAD cells to treat patients with atherosclerotic CLI with minor tissue loss (Rutherford Category 5) up to the ankle level, who are unsuitable for revascularization or carry an unfavorable risk-benefit for that treatment. The study will

enroll 246 patients, who after screening are randomized in a ratio of 2:1 to treatment with intramuscular injections of PLX-PAD 300X10^6 cells or placebo at two occasions, 8 weeks apart. The primary efficacy endpoint is time to major amputation or death (amputation free survival), which will be assessed in follow-up of after at least 12 months and up to 36 months.

Conclusions: Based on favorable pre-clinical and initial clinical study results, the PACE phase III randomized controlled trial will evaluate placenta-derived PLX-PAD cell treatment in patients with critical limb ischemia, carrying an unfavorable risk-benefit for revascularization.

Abstract word count: 255

Key words: Cell therapy, critical limb ischemia, trial design

Introduction

Critical limb ischemia (CLI) constitutes the most advanced stage of chronic peripheral arterial disease (PAD) and includes rest pain and ischemic foot lesions. The condition affects 1-5 % of all PAD patients, which corresponds to an incidence of 500-1000 / million population per year (1). Overall, the prevalence of PAD increases worldwide, most remarkably in low- and middle income countries (2). Major amputation and death are the ultimate consequences of CLI, and a 1-year amputation rate of 15-25% is commonly reported, while amputation & mortality rate ranges 30-40%. The single evidence-based recommendation for treatment is revascularization (1,3,4). Due to co-morbidities with greater risk to perform an interventional procedure, or based on anatomical or technical issues, a proportion of CLI patients is not reasonable to revascularize or to re-revascularize after a failed procedure. Few treatments exist for such “poor-option” cases. Prostanoid therapy has been reasonably well studied in randomized controlled trials, but does not carry evident effect, and is not recommended in present guidelines (1,3). Since about 20 years, therapeutic angiogenesis has been studied, based on either gene or cell therapy.

Gene therapy

Gene therapy utilizing growth factors, Vascular Endothelial Growth Factor (VEGF), Fibroblast Growth Factor (FGF) and Hepatocyte Growth Factor (HGF), has been investigated in mostly smaller clinical trials, with varying success with regard to the major efficacy endpoint, amputation-free survival (AFS). Only NV1FGF has been investigated in a larger randomized placebo controlled trial, TAMARIS (5) that did not show any better outcome regarding survival or major amputation in the treatment group compared to placebo, despite the fact that a former, smaller trial, TALISMAN (6), showed that major amputation, as a secondary

endpoint, was significantly less frequent among NV1FGF treated subjects. Injections of the HGF plasmid have yet to prove efficacy with regard to major events, though smaller randomized placebo controlled trials have shown reduced rest pain (7) and increased toe brachial index (TBI) (8) at follow up. More has to be learnt both from basic and clinical research to possibly adopt effective gene therapy for PAD (9), though Iver and Annex (10) discuss a conceivable end of gene therapy trials, based on the lack of an evident break-through.

Cell therapy

The potential benefit of cell therapy is that cell secretion is multifactorial and therefore not based solely on a single growth factor. Initiated by a Japanese study (11) comparing bone-marrow- and peripheral blood mononuclear cells (PBMC) injected into the limb muscles of patients with PAD, several cell-based studies have been performed, specifically in CLI patients with no option for revascularization. The Japanese study (11) showed improved ankle-brachial index (ABI) and transcutaneous tissue oxygen pressure ($TcPO_2$) and reduced rest pain in the bone-marrow mononuclear cell treated group. Though the majority of studies have utilized intramuscular injections of the growth factor, the largest trial, Juventas, treated 160 patients with intra-arterial infusions of bone-marrow mononuclear cells (BM-MNC) compared to placebo (12). At 6 months there was no difference in the rate of major amputations.

In a meta-analysis by Teraa et al (13), including 12 randomized controlled trials (RCT) in autologous cell therapy for CLI, major amputations were significantly reduced. Most importantly, when only placebo controlled RCTs were included, the major amputations were no longer significantly reduced, indicating the importance of placebo controlled trials in cell

144 therapy. In a later meta-analysis by the same author group (14) only including placebo-
145 controlled RCTs, this outcome was verified. Recently this finding was also verified in another
146 meta-analysis on CD34+ mononuclear cell therapy (CD34+MCT), including 10 trials (15). Total
147 amputations and ulcer healing were reduced in comparison with findings in the placebo
148 treated groups. Major amputation and survival were, however, not significantly reduced.
149 This publication also concluded the beneficial value of a high CD34+ cell content.
150
151 **Autologous or allogeneic cell utilization**
152 From an immunological point of view, autologous cell treatment may theoretically provide
153 an immunological advantage. Nevertheless, it has been shown that cells harvested from
154 older individuals, and in particular those with cardiovascular risk factors or critical limb
155 ischemia, are reduced in number and functionality (16, 17). Furthermore, harvesting
156 autologous cells from bone marrow involves an invasive procedure, while peripheral blood
157 utilization requires granulocyte colony stimulation factor (G-CSF) treatment that potentially
158 may cause harm due to the high white blood cell content that is developed (18). Allogeneic
159 MSCs have been shown to exhibit low immunogenicity (19), thus, utilizing allogeneic
160 younger, more potent cells, rather than treatment with cells harvested from the diseased
161 patients themselves, therefore should be of benefit. In this respect PLX-PAD cells from young
162 healthy placental tissue have the potential for higher efficacy than previously seen with
163 autologous cell products.
164
165 **PLX-PAD: Allogeneic Cell Therapy**
166 PLX-PAD is a cell therapy product, composed of placental expanded adherent stromal cells.
167 While PLX-PAD cells exhibit membrane marker expression typical of classical mesenchymal

168 stromal cells (20), they have a minimal ability to differentiate in vitro into cells of
169 mesodermal lineage. Therefore, their proposed mechanism of action is a timely secretion of
170 various proteins which induce angiogenesis, immunomodulatory activities, and promotion
171 of regeneration of muscle tissue.
172 Angiogenesis, the formation of new vessels, is induced by a variety of factors released from
173 ischemic tissues, and is a critical physiological mechanism for alleviation of PAD or for
174 recovery of muscle tissue functionality after injury. The angiogenic process involves
175 migration of endothelial progenitors and pericytes towards the site of interest. *In vitro*
176 studies have shown the capacity of PLX-PAD cells to promote endothelial cell proliferation
177 (20). The cells secrete pro-angiogenic proteins including VEGF, angiopoietin-1, osteopontin,
178 MMP-1, MMP-2, HGF and angiogenin, all of which are up-regulated under hypoxic culture
179 conditions (20, 21 and unpublished data). Angiogenin further interacts with endothelial and
180 smooth muscle cells, resulting in cell migration, invasion, proliferation and formation of
181 tubular structures (22). (Fig 1, Table 1).
182 PAD is associated with an inflammatory process that leads to tissue damage and precludes
183 active repair. Oxidative stress due to endothelial dysfunction is evident in PAD and leads to
184 persistent inflammation. Proinflammatory cytokines, e.g. TNF-α, IL-6 , IL-1β , play a key role
185 in the inflammatory process, and PLX-PAD cells mitigate this process by releasing anti-
186 inflammatory and immunomodulating cytokines (i.e. GDF-15, CXCL12,TGF-β). Following
187 exposure to pro-inflammatory cytokines (such as TNF-α and IFN-γ) PLX-PAD cells further
188 upregulate some of the anti-inflammatory secretions (i.e. IDO, PD-L1, HGF, IL-11, CCL5).
189 Furthermore, when cultured with activated PBMCs, PLX-PAD induce upregulation of PBMC
190 secreted anti-inflammatory cytokines such as IL-10, and IL-1RA), also indirectly affecting
191 endothelial dysfunction and protecting endothelial cell viability (20 and unpublished data).

As ischemic conditions lead to muscle degeneration, muscle regeneration is of potential therapeutic benefit in PAD. PLX-PAD cells have been shown to promote migration of skeletal muscle cells *in vitro* and improve muscle function and accelerate muscle regeneration in vivo (manuscript in preparation).

To summarize, PLX-PAD cells secrete proteins that are known to be involved in promoting angiogenesis, downregulating inflammation and inducing regeneration of muscle tissue.

In vivo, in the mouse hind limb ischemia (HLI) model in which the femoral artery of one hindlimb is cut and ligated thus inducing complete ischemia in the operated limb, (21, 23), PLX-PAD cells have been shown to restore blood flow to the ischemic limb . Furthermore, it was shown that PLX-PAD cells exert a systemic effect, since injection of the cells to the contralateral limb exerted an almost similar restoration of blood flow, but required a larger dose of cells. A second administered dose of PLX-PAD cells 21 days after the first dose afforded additional efficacy in re-establishing blood flow in case the effect was declining (Fig 2). This study and others have also shown that PLX-PAD cells injected intramuscularly do not migrate from the injection site to other tissues and do not differentiate in culture, further supporting the suggested mode of action of PLX-PAD cells through secretion of proteins.

Clinical studies in PAD

Two phase I open-label, dose escalation studies were conducted to assess the safety of intramuscular injections of PLX-PAD cells in 27 CLI subjects (Rutherford Categories 4 and 5), who were not candidates for revascularization.

Study 1202-1 was conducted in Germany and assessed three single doses of 175 million cells (low dose, n=3), 315 million cells (intermediate dose, n=6) and 595 million cells (high dose, n=6). Study 1202-2 was conducted in the United States (US) and assessed a single versus 2 doses (2 weeks apart) of 280 million cells, the first group included 7 patients, the latter included 5 patients. PLX-PAD cells were administered intramuscularly into the affected leg via 30 to 50 injections.

Overall, the safety of this process in CLI subjects was found to be acceptable, and it was confirmed that HLA–matching is not required. Adverse events included mostly injection-sites reactions such as pain, muscle contractions/fasciculations, pruritus, hematoma, etc. (mostly transient and of mild/moderate intensity), transient allergic reactions, and bad breath due to the DMSO (dimethyl sulfoxide) content.

These phase I studies were not powered to demonstrate clinical efficacy, however, some parameters have indicated a positive clinical effect. The pooled amputation free survival rate at 6 months and 1 year across the two studies was 96% and 85% respectively, which is higher than the rates described in similar patient populations (24, 25). Pain scores, as assessed by the Visual Analog Scale (VAS), showed a trend of decrease after treatment with PLX-PAD in all dose groups, up to a decrease of 2.5 units in the patients treated at the dose of 315 million cells. $TcPO_2$, which is considered an indicator of tissue perfusion, demonstrated a trend of increase over time in all study groups with the greatest increase of up to 15 mmHg in the repeated-dose group (Fig 3). (data on file)

In summary, based on the pro-angiogenic, immunomodulatory, and muscle regeneration capacities of PLX-PAD, as well as the results from animal experiments and outcome of the clinical studies in PAD patients, a phase III trial was designed.

PACE trial design

The PACE study (*A randomized, double-blind, multicenter, placebo-controlled, parallel-group Phase III study to evaluate the efficacy, tolerability and safety of intramuscular injections of PLX-PAD for the treatment of subjects with critical limb ischemia (CLI) with minor tissue loss who are unsuitable for revascularization*) was designed to investigate time to major amputation or death (AFS) after up to 36 months. The study is planned to enroll a total of 246 patients with minor foot lesions (Rutherford Category 5) up to the ankle level. Patients should be unsuitable for revascularization or carry an unfavorable risk- benefit to revascularization. Ineligibility for revascularization is determined by either severe co-morbidity, anatomical or technical challenges (e.g. lack of vein for a bypass or inadequate target vessels for an endovascular procedure) or failed revascularization procedures with persistence of CLI after the procedure. Only patients with atherosclerotic disease are included, those with thrombangitis obliterans (Buerger´s disease) are excluded. Table 2 shows the main inclusion and exclusion criteria.

Subjects are screened up to 5 weeks before randomization. If found eligible, patients are randomized in a ratio of 2:1 to treatment with PLX-PAD $300x10^6$ cells or with placebo. Treatment is administered at two time points, 8 weeks apart. At each occasion, thirty intramuscular injections, 0.5 mL each, are administered in the index leg along its length, anteriorly and posteriorly, according to a standard injection-sites scheme. A strict procedure is applied for cell preparation and administration in order to maintain study blinding. Dosage and timing of injections are based on preclinical and accumulated clinical data.

Each subject will be followed-up for at least 12 months post randomization or until the 12 months visit of the last patient randomized. Maximal follow up allowed by protocol is 36

months post randomization, hence all subjects will be followed-up for 12-36 months. The study design is presented in Fig. 4.

The primary efficacy endpoint of the study is time-to occurrence of major amputation or death, i.e. amputation-free survival up to 36 months after randomization. Safety and tolerability are to be evaluated as well as other secondary and exploratory endpoints (Table 3). The study will also assess a potential economic benefit of this regenerative treatment approach by applying a health-economic evaluation, taking into account relevant parameters as days of hospitalization and patient reported quality of life.

The study will be performed in 50 sites in Europe and the USA

Statistical considerations

The sample size of 246 subjects provides a power of 89.7%, and is based on the 2:1 ratio randomization to treatment, an estimated AFS of 65% in the placebo group at the end of the first year, and a risk reduction of approximately 50% for the PLX-PAD group during the first year, using the the log-rank test. The primary endpoint will be analyzed using the Cox Proportional Hazards model. The study randomization is stratified for the presence of diabetes mellitus, for the extent of ischemic lesions, and for geographical region, which will be covariates in the statistical model.

Discussion

Although critical limb ischemia affects a small proportion of patients with PAD, and an increasing part of them are offered revascularization (26), other treatments are required for some patients in order to possibly increase survival and reduce major amputations. The fact that trials have had problems with slow recruitment of no-option patients, e.g. the TAMARIS

trial (5) and the AGILITY HGF trial, that had to be canceled for that reason (10) might be interpreted in a way that few patients do require alternative treatments. However, in addition to no-option cases, revascularizations may fail or only partly reduce CLI symptoms, and poor option subjects for revascularization due to co-morbidity or for technical reasons will still be a reality. In a recent paper, Martinez et al (27) discussed predictive factors of poor short-term outcome (mortality and major amputation) following revascularization, including age, low hemoglobin values, acute myocardial infarction, ischemic ulcers and infrapopliteal revascularization. For such groups of fragile CLI patients, therapeutic angiogenesis may be an alternative.

As larger gene therapy trials have failed, although there is still an interest in the evaluation of HGF (9), and doubts exist with regard to cell therapy (14,15), no such treatment has yet been approved for clinical use. It could be interpreted that single growth factor trials may not be able to provide the complete array of factors that the patients in this population require. Therefore, precursor cell therapy would potentially provide a more complete array of factors. It is reasonable to assume that the age and condition of cells, harvested from the potential patients, are crucial. It has been shown that CLI patients produce lower levels of progenitor cells (17) and an increasing cardiovascular risk is also related to a lower number of progenitor cells (16). In addition, cells harvested and injected at the point of service, are not by their nature able to be characterized nor quantified before being injected, therefore bringing into question their very nature. Furthermore, it has been shown that growth of isolated mesenchymal stem cells is significantly related to the age of the donor (28), and thus young allogeneic placental cells may be most relevant for the purpose of treatment as they come from a young healthy donor.

Most importantly, PLX-PAD cells, being of a placental source, known for its immune-priviledged characteristics, have been shown to not exert an immunological effect neither in vitro nor in vivo in animal models and humans, requiring no immunesupression prior to PLX-PAD administration (29).

The PACE trial only includes patients with ischemic lesions and does not enroll Rutherford Category 4 cases with just rest pain, due to the inobjectivity of evaluating pain. In practice, CLI patients with rest pain may also be those who most frequently will be offered revascularization. Hence, pain is not included in the composite primary efficacy endpoint in Rutherford Category 5 patients. Furthermore, these patients are at higher risk of major amputation, thus providing the best evidence on the effect on AFS.

The trial design takes into account the greater efficacy of two cell administrations rather than one as shown in both animal models and human subjects, and therefore a second administration session is given two months after the first session. Some patients will be followed up to 36 months, which will enable collection of highly important information on long-term effects of the treatment. and will also increase knowledge on the natural course of severe CLI . The primary efficacy endpoint, amputation free survival is selected as the strictest endpoint to be evaluated. Disease progression, wound healing, ischemic pain, quality of life, $TcPO_2$, ABI/TBI measures and hospitalization days data are included as secondary and exploratory endpoints.

The term therapeutic angiogenesis may be interpreted as the mode of action by which new vessels are formed, thus potentially increasing perfusion. In human studies, however, present imaging technology is only occasionally able to show newly developed vessels despite the fact that subjects may be improved. It is evident that other pathophysiological events are affected as well, primarily the inflammatory process. PLX-PAD cells exert effects

on both angiogenesis and tissue inflammation, but also on regeneration of muscle cells. Whether the latter is a mechanism of value for improvement of function and symptoms in CLI patients should be further investigated.

In summary, cell therapy works in a multifactorial way, PLX-PAD cells are young and potent, they secrete relevant factors, are easily accessible in required quantity without harvesting from fragile patients putting those at additional risk and have shown pre-clinical and initial clinical evidence of efficacy. The design of the PACE trial, including only patients with ischemic foot lesions, dual injections along the whole limb, follow-up up to 36 months, and with a primary efficacy endpoint based on long term time-to-event regarding amputation-free survival may allow for better understanding of perfusion enhancement and change of inflammatory response and improved outcome for patients with severe critical limb ischemia.

***Declaration of interest*:** L Norgren, N Weiss, S Nikol, RJ Hinchliffe, JC Lantis, MR Patel and H Reinecke are members of the Steering Committee for the PACE trial. R Ofir, Y Rosen, D Peres and Z Aberman are employees of Pluristem Ltd

Conflicts of Interest

L Norgren: Consultations, advisory boards and/or research grants: AnGes, AstraZeneca, Bayer, CESCA, Mitsubishi, Pluristem

N Weiss: Consultations, advisory boards and/or research grants: Amgen, Bard, Bayer, Fresenius, Merck, Pfizer, Pluristem, Terumo

S Nikol: Consultations: Pluristem

RJ Hinchliffe: Nothing to declare

JC Lantis: Consultations: Pluristem

MR Patel: Advisory boards: Bayer, Jansen. Research grants: Pluristem, Bayer, Jansen, AstraZenca

H Reinecke : Consultations: BMS, MedUpdate, NephroUpdate, Pfizer, Pluristem. Grants: German Federal Ministry for Education and Research, Bard, Bayer, Biotronic

References

1.Norgren L, Hiatt WR, Dormandy JA, Nehler MR, Harris KA, Fowkes FG. Inter-Society Consensus for the Management of Peripheral Arterial Disease (TASC II). Eur J Vasc Endovasc Surg 2007;33:S1-S75.

2.Fowkes FG, Rudan D, Rudan I, Aboyans V, Denenberg JO, McDermott MM et al. Comparison of global estimates of prevalence and risk factors for peripheral artery disease in 2000 and 2010: a systematic review and analysis. Lancet 2013; 382:1329-40

3.Gerhard-Herman MD, Gornik HL, Barrett C, Barshes NR, Corriere MA, Drachman DE, et al. 2016 AHA/ACC Guideline on the Management of Patients With Lower Extremity Peripheral Artery Disease: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. Circulation 2017;135:e726-e779.

4.Aboyans V, Ricco J-B, Bartelink EL, Bjorck M, Brodmann M, Cohnert T et al. 2017 ESC guidelines on the diagnosis and treatment of peripheral arterial diseases, in collaboration with the European Society for Vascular Surgery (ESVS). Eur J Vasc Endovasc Surg. 2018; 55:305-368

5.Belch J, Hiatt WR, Baumgarnter I, Driver IV, Nikol S, Norgren L et al. Effect of fibroblast growth factor NV1FGF on amputation and death: a randomized placebo-controlled trial of gene therapy in critical limb ischemia. Lancet 2011; 377:1929-37

6.Nikol S, Baumgartner I, Van Belle E, Diehm C, Visona A, Capogrossi MC et al. Therapeutic angiogenesis with intramuscular NV1FGF improves amputation-free survival in

patients with critrical limb ischemia. Mol Ther 2008; 16:972-8

7.Shigematsu H, Yasuda K, Iwai T, Sasajima T, Ishimaru S, Ohashi Y et al. Randomized, double-blind, placebo-controlled clinical trial of hepatocyte growth factor plasmid for critical limb ischemia. Gene Ther 2010; 17:1152-61

8.Powell RJ, Goodney P, Mendelsohn FO, Moen EK, Annex BH, HGF-205 Trial Investigators. Safety and efficacy of patient specific intramuscular injection of HGF plasmid gene therapy on limb perfusion and wound healing in patients with ischemic lowere extremity ulceration: results of the HGF-0205 trial. J Vasc Surg 2010; 52:1525-30

9.Sanada F, Taniyama Y, Muratsu J, Otsu R, Shimitzu H, Rakugi H et al. Gene-therapeutic strategies targeting angiogenesis in peripheral artery disease. Medicines 2018; 30: 5(2). doi: 10.3390/medicines5020031

10.Iver SR, Annex BH. Therapeutic angiogenesis for peripheral artery disease. JACC Basic Trans Sci 2017; 2:503-12

11.Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U,Shintani S, Masaki H et al. Theraputic angiogenesis for patients with critical limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002; 360:427-35

12.Teraa M, Sprengers RW, Schutgens RE, Slaper-Cortenbach I, van der Graaf Y, Algra A et al. Effect of repetetive intra-arterial infusion of bone marrow mononuclear cells in patients with no-option limb ischemia. The randomized, double-blind, placebo-controlled rejuvenating endothelial progenitor cells via transcutaneous intra-arterial supplementation. Circulation 2015; 131:851-60

13.Teraa M, Sprengers RW, van der Graaf Y, Peters CE, Moll FL, Verhaar MC. Autologous bone marrow derived cell therapy in patients with critical limb ischemia: a meta-analysis of randomized controlled clinical trials. Ann Surg 2013; 258:922-9

14.Peeters Weem SM, Teraa M, De Borst GJ, Verhaar MC, Moll FL. Bone marrow derived cell therapy in critical limb ischemia: a meta-analysis of randomized placebo controlled trials. Eur J Vasc Endovasc Surg 2015; 50:775-83

15. Pan T, Wei Z, Fang Y, Dong Z, Fu W. Therapeutic efficacy of CD34+ cell-involved mononuclear cell therapy for no-option critical limb ischemia: a meta-analysis of randomized controlled clinical trials. Vasc Med 2018; doi: 10.1177/1358863X17752556 (Epub ahead of print)

16. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA Quyyumi AA et al. Circulating endothelial progenitor cells, vascular function and cardiovascular risk. New Eng J Med 2003; 348:593-600

17. Teraa M, Sprengers RW, Westerweel PE, Gremmels H, Goumans MJ, Teerlink T et al. Bone marrow alteration and lower endothelial progenitor cell numbers in critical limb ischemia patients.
PLoS One 2013; 8: e55592 doi:10.137/journal.pone.0055592. Epub 2013

18. Jonsson TB, Larzon T, Arfvidsson B, Tidefeldt U, Axelsson CG, Jurstrand M et al. Adverse events during treatment of critical limb ischemia with autologous peripheral blood mononuclear cell implant. Int Angiol 2012; 31:77-84

19. Zhang J, Huang X, Wang H, Liu X, Zhang T, Wang Y, Hu D. The challenges and promises of allogeneic mesenchymal stem cells for use as a cell-based therapy. Stem Cell Res Ther. 2015 Dec 1;6:234.

20. Roy R, Brodarac A, Kukucka M, Kurtz A, Becher PM, Jülke K et. al. Cardioprotection by placenta-derived stromal cells in a murine myocardial infarction model. J Surg Res. 2013 Nov;185(1):70-83.

21 Zahavi-Goldstein E, Blumenfeld M, Fuchs-Telem D, Pinzur L, Rubin S, Aberman Z, Sher N, Ofir R. Placenta-derived PLX-PAD mesenchymal-like stromal cells are efficacious in

452 rescuing blood flow in hind limb ischemia mouse model by a dose- and site-dependent
453 mechanism of action. Cytotherapy. 2017 Dec;19(12):1438-1446.
454 22. Gao X, Xu Z. Mechanisms of action of angiogenin. Acta Biochim Biophys Sin(Shanghai).
455 2008 Jul;40(7):619-24.
456 23 Prather WR, Toren A, Meiron M, Ofir R, Tschope C, Horwitz EM. The role of placenta
457 derived adherent stromal cell (PLX-PAD) in the treatment of critical limb ischemia.
458 Cytotherapy. 2009;11(4):427-34.
459 24 Van Belle E, Nikol S, Norgren L, Baumgartner I, Driver V, Hiatt WR, Belch J. Insights on
460 the role of diabetes and geographic variation in patients with critical limb ischaemia.
461 Eur J Vasc Endovasc Surg. 2011 Sep;42(3):365-73.
462 25. Reinecke H, Unrath M, Freisinger E, Bunzemeier H, Meyborg M, Lüders F, Gebauer K,
463 Roeder N, Berger K, Malyar NM. Peripheral arterial disease and critical limb ischaemia: still
464 poor outcomes and lack of guideline adherence. Eur Heart J. 2015 Apr 14;36(15):932-8.
465 26.Hong MS, Beck AW,Nelson PR. Emerging national trends in the management and
466 outcomes of lower extremity peripheral arterial disease. Ann Vasc Surg 2011; 25:44-54
467 27.Martinez M, Sosa C, Velescu A, Llort C, Elosus R Clara A. Predicitve factors of a poor
468 outcome following revascularization for critical limb ischemia: implications for practice.
469 Int Angiol 2018; doi:10.23736/S0392-9590.18.03986-X (E-pub ahead of print)
470 28. Beane OS, Fonseca VC, Cooper LL, Koren G, Darling EM. Impact of aging on the
471 regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal
472 stem/stromal cells. PLoS One. 2014 Dec 26;9(12):e115963.
473 29. Consentius C, Akyüz L, Schmidt-Lucke JA, Tschöpe C, Pinzur L, .Ofir R et.al
474 Mesenchymal Stromal Cells Prevent Allostimulation In Vivo and Control Checkpoints of Th1
475 Priming: Migration of Human DC to Lymph Nodes and NK Cell Activation.
476 Stem Cells. 2015 Oct;33(10):3087-99. doi: 10.1002/stem.2104.
477

Figure 1

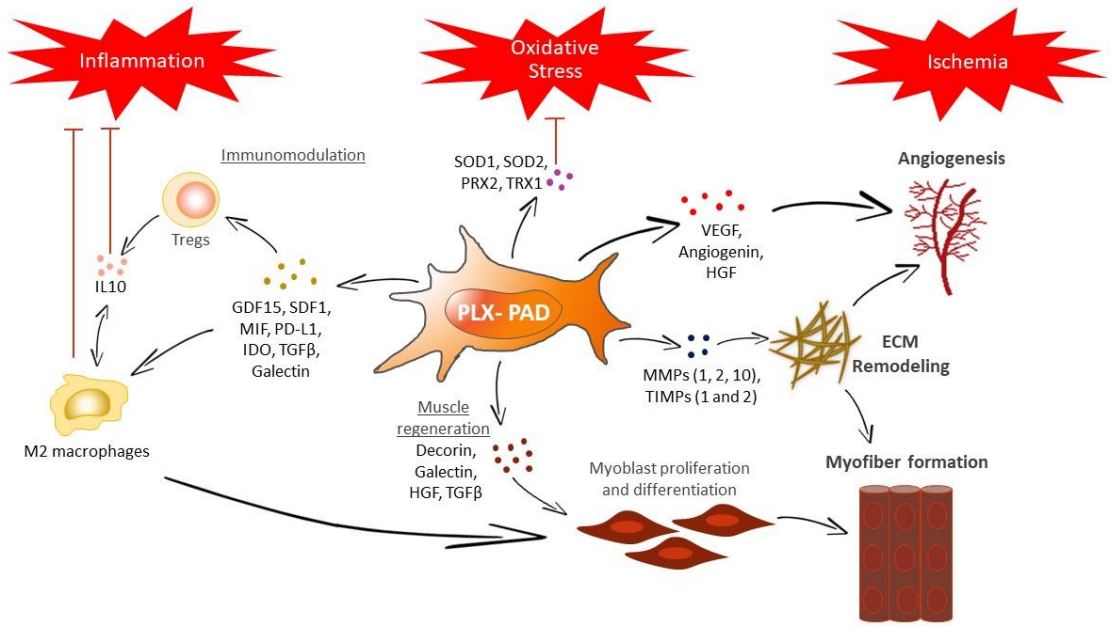
Inflammation
Oxidative Stress
Ischemia
Immunomodulation
SOD1, SOD2, PRX2, TRX1
Angiogenesis
Tregs
IL10
GDF15, SDF1, MIF, PD-L1, IDO, TGFβ, Galectin
PLX- PAD
VEGF, Angiogenin, HGF
ECM Remodeling
MMPs (1, 2, 10), TIMPs (1 and 2)
M2 macrophages
Muscle regeneration
Decorin, Galectin, HGF, TGFβ
Myoblast proliferation and differentiation
Myofiber formation

Figure 2

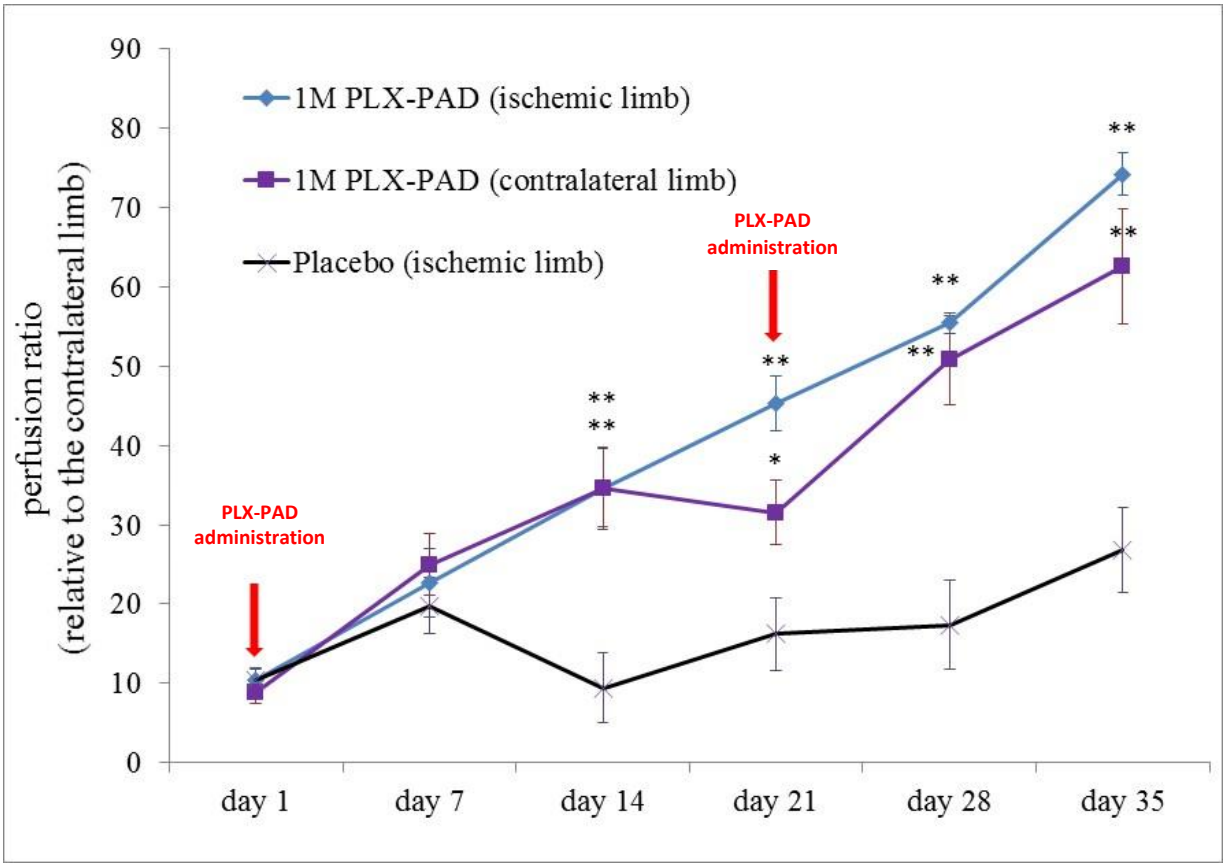
90
80
70
60
50
40
30
20
10
0
perfusion ratio
(relative to the contralateral limb)
1M PLX-PAD (ischemic limb)
1M PLX-PAD (contralateral limb)
Placebo (ischemic limb)
PLX-PAD
administration
PLX-PAD
administration
**
**
**
**
**
**
*
day 1
day 7
day 14
day 21
day 28
day 35

Figure 3

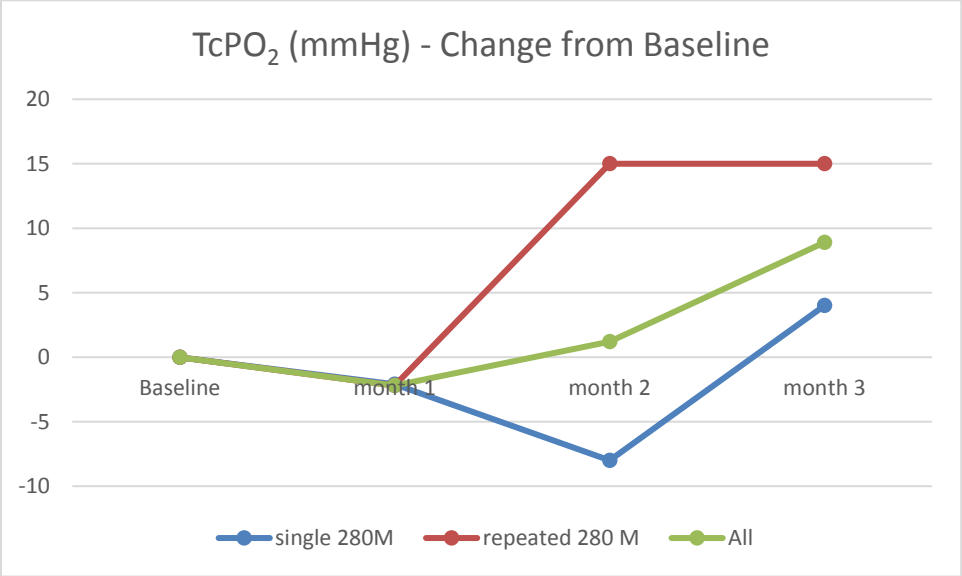
TcPO2 (mmHg) - Change from Baseline
20
15
10
5
0
-5
-10
Baseline
month 1
month 2
month 3
single 280M
repeated 280 M
All

Figure 4

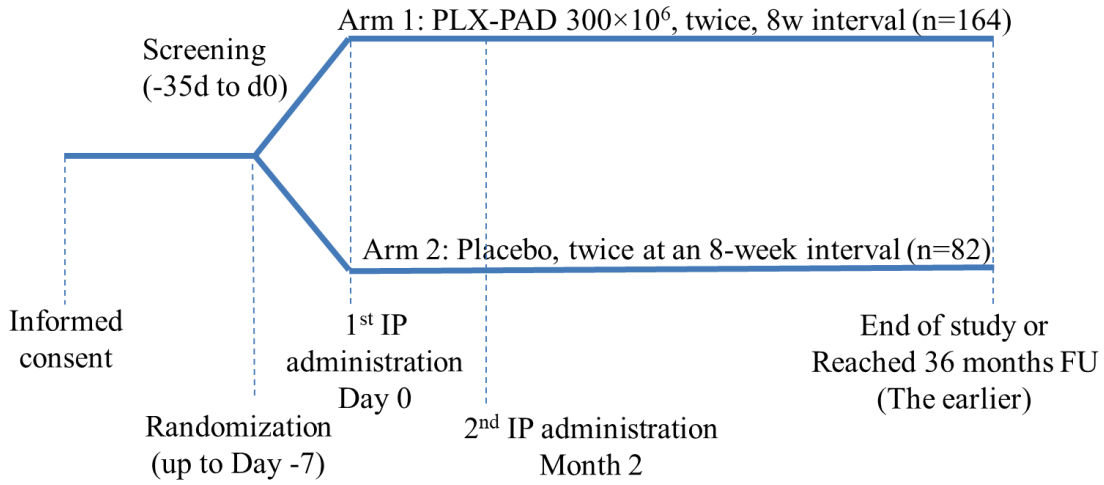
Arm 1: PLX-PAD 300×10^6, twice, 8w interval (n=164)
Screening
(-35d to d0)
Arm 2: Placebo, twice at an 8-week interval (n=82)
Informed
consent
1st IP
administration
Day 0
End of study or
Reached 36 months FU
(The earlier)
Randomization
(up to Day -7)
2nd IP administration
Month 2

Table 1

Cytokines secreted by PLX-PAD and their function

Angiogenesis	VEGF (Vascular Endothelial Growth Factor)
	Angiogenin
	Angiopoietin 1
	HGF (Hepatocyte Growth Factor)
	Osteopontin
	MMP-1 (matrix metalloproteinase 1)
	MMP-2
Immunomodulation	Osteopontin
	CXCL12 /SDF 1 (Stromal Cell-derived Factor 1)
	GDF 15 (Growth Differentiation Factor 15)
	MIF (Macrophage Migration Inhibition Factor)
	IDO (Indoleamine 2,3-dioxygenase)
	TGF-β (Transforming growth factor beta)
	PD-L1 (Programmed death ligand 1)
	HGF
	IL-11 (Interleukin 11)
	CCL5 (RANTES- regulated on activation, normal T cell expressed and secreted)
Muscle regeneration	Decorin
	MMP 1
	HGF
	TGF β
	Galectin 1
	IGFBP-3 (Insulin growth factor binding protein 3)
	FLRG (FSTL3- Follistatin-related protein 3)
	Osteopontin
	CXCL12 /SDF 1

Table 2

Main inclusion criteria:

- Age 45-99 years.
- CLI due to atherosclerosis with minor tissue loss (Rutherford 5) up to the ankle level.
- Ankle pressure ≤70 mmHg or toe pressure ≤50 mmHg.
- Subject unsuitable for revascularization (by any method) in the index leg, based on unfavorable risk-benefit assessment.
- Ischemic lesions neither healing, nor significantly worsening (within 2 weeks during screening)
- Ischemic lesions without tendon or bone exposure (unless secondary to a minor amputation).

Main Exclusion criteria:

- Non-atherosclerotic PAD (e.g. Buerger's disease).
- CLI with major tissue loss (Rutherford Category 6) in either leg.
- Evidence of active infection (e.g., cellulitis, osteomyelitis).
- Subject having undergone surgical revascularization <1 month prior to study, or endovascular revascularization/minor amputation <2 weeks prior.
- Planned or potential need for major/minor amputation or revascularization within 1 month of study entry.
- Aorto-iliac stenosis or common femoral artery stenosis ≥70%.
- Use of hyperbaric oxygen therapy, prostanoids, spinal cord stimulation, lumbar sympathectomy, wound dressing containing cells or growth factors, or topical platelet derived growth factor.
- Stroke or acute myocardial infarction/unstable angina within 3 months prior to screening.
- Severe congestive heart failure symptoms (New York Heart Association [NYHA] Stage IV).
- Uncontrolled severe hypertension.
- Diabetes mellitus with HbA1c >10%.
- Subject on renal replacement therapy or with eGFR <15 mL/min/1.73m^2.
- Pulmonary disease requiring supplemental oxygen treatment on a daily basis.
- Active malignancy or history of malignancy within 5 years prior to study entry.

Table 3

Primary Efficacy Endpoint:

- Time to occurrence of major amputation or death (amputation-free survival).

Main Secondary and Exploratory Endpoints:

- Time to first occurrence of any of the following single events:
  - o Major amputation of the index leg.
  - o Revascularization due to worsening of CLI in the index leg.
  - o Doubling of total ulcer area from baseline in the index leg.
  - o De novo necrosis in the index leg.
  - o All-cause mortality.
- Time to major amputation of the index leg.
- Complete healing of all ischemic lesions at 12 months.
- Change from baseline in ischemic pain (Numerical rating scale (NRS)) at 6 months.
- Time to death or major amputation or adjudicated major amputation of the index leg.
- Time to all cause death.
- Decrease of 50% or more in total ulcer area at 6 months.
- Complete healing of all ischemic lesions in the contralateral leg.
- Time to occurrence of major amputation of the contralateral leg.
- Change in health- and disease-related Quality of Life at 12 months.
- Changes in $tcPO_2$, ankle-brachial index (ABI), toe-brachial index (TBI).
- Revascularization procedure in the index leg within 12 months from treatment.
- Hospitalization days.
- Change from baseline in plasma cytokine levels after PLX-PAD administration.
- Change from baseline in mRNA expression profile after PLX-PAD administration.

Legends to figures

Figure 1

Suggested mechanism of PLX-PAD effect in CLI. PLX-PAD secretions can mitigate CLI pathology by simultaneously affecting several disease associated pathways. PLX-PAD secrete immunomodulatory cytokines which support the induction of M2 macrophages and elevate the level of circulating regulatory T cells, leading to elevation in IL-10 and resolution of inflammation. In addition, PLX-PAD secrete factors which directly support angiogenesis and muscle regeneration. These processes are further supported by the PLX-PAD secretion of enzymes with antioxidant activity, which can protect blood vessels from oxidative damage, and the secretion of ECM (extracellular matrix) remodeling enzymes which enable regeneration.

Figure 2

PLX-PAD cells are effective in re-establishing blood flow in the HLI mouse model. Intramuscular (IM) administration to the ischemic or contralateral limb, were effective in rescuing blood flow to the ischemic limb compared to placebo control. PLX-PAD were administered 1 and 21 days (depicted by arrows on graph) following induction of HLI. n=10 for each PLX-PAD treated group and n=5 for placebo group. $F(39,70)=30.82$, $p<0.0001$. Blood flow is measured as perfusion ratio relative to the contralateral limb. *$p<0.05$; *** $p < 0.0001$, compared to placebo control.

Figure 3

Change of TcPO2 in Study 1202-2. M=million cells

Figure 4

Study design, timing of injections and follow up.

794
795
796
797
798
799
800
801
802